COMPARATIVE STUDY OF THE ANTIBACTERIAL PROPERTIES OF ASCORBIC ACID AND REDUCTOGENIC COMPOUNDS[1]

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Ascorbic acid has an antibacterial effect against tubercle bacilli (Boissevain and Spillane, 1937; Leitner, 1937; Sirsi, 1952) and many other microorganisms (von Gagyi, 1936; Grootten and Bezssonoff, 1935; Lwoff and Morel, 1942*a*; Ehrismann, 1942). The mechanism of action and the chemical group responsible for the bacterial inhibition are not known.

Bactericidal effect due to a lowering of the pH has been suggested by von Gagyi (1936) and Ehrismann (1942); however, Slade and Knox (1950) have found ascorbic acid to be bacteriostatic for a group A hemolytic streptococcus even though the pH was near neutrality. Ehrismann (1942) found that anaerobes were generally stimulated by ascorbic acid, whereas strict aerobes were generally inhibited, suggesting that in the latter inhibition was due to a reduction in the O/R potential of the medium. Lwoff and Morel (1942*b*) found that inhibition of *Proteus vulgaris* by ascorbic acid was counteracted by the presence of reducing agents and by substances which catalyzed the breakdown of hydrogen peroxide. Similar results were obtained with *Escherichia coli* and *Bacillus subtilis*. They concluded that the inhibition was due to hydrogen peroxide formed during the auto-oxidation of ascorbic acid.

Myrvik *et al.* (1954) reported that some auto-oxidized derivative of ascorbic acid was most likely the active principle against mycobacteria. The addition of catalase in the form of rabbit red blood cell lysates failed to influence the inhibition. Furthermore, they were unable to associate any appreciable tuberculostatic activity with any of the classical breakdown products of ascorbic acid such as 2,3-diketogulonic acid, oxalic acid, furfural, and threonic acid. The active principle was heat-stable, water soluble, and sparingly soluble in 95 per cent alcohol but was insoluble in acetone, ether, petroleum ether, benzene, carbon tetrachloride, and chloroform.

The present investigation was an attempt to determine the chemical group responsible for the antibacterial properties of ascorbic acid solutions. Evidence is presented which indicates that these inhibitory properties reside in the oxidized enediol (diketone) group.

MATERIALS AND METHODS

Microorganisms used and source. BCG strain of *Mycobacterium tuberculosis* var. *bovis* and *M. phlei:* Dr. R. S. Weiser, Department of Microbiology, University of Washington, School of Medicine, Seattle, Washington. Ravenel strain of *M. tuberculosis* var. *bovis* and H37Rv strain of *M. tuberculosis* var. *hominis:* Mr. W. Steenken, Jr., Trudeau Laboratory, Trudeau, New York. *Escherichia coli* and *Pseudomonas* sp.: Culture collection, Department of Microbiology, University of Virginia, School of Medicine, Charlottesville, Virginia.

The mycobacteria were maintained at 37 C on Youmans' modification (1946) of Proskauer and Beck's broth. Inocula were prepared by grinding a portion of the surface growth in a sterile mortar and diluting with sterile saline. The other microorganisms were maintained at 37 C in nutrient broth (Difco).

Antibacterial tests. The antibacterial tests were conducted using Youmans' (1946) modification of Proskauer and Beck's medium containing 5 per cent Seitz filtered bovine serum. The compounds to be tested were dissolved in distilled water, adjusted to pH 7.0, sterilized by filtration through a Swinny filter, and serially double diluted in one ml amounts of the above medium. Each tube was inoculated with one drop of a bacterial suspension containing approximately 10^{-3} mg of wet weight standardized in a Coleman nephelometer model 9. Cultures of the slow growing mycobacteria were incubated at 37 C

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TABLE 1

*Comparative antibacterial spectra of ascorbic acid, reductogenic compounds and alpha diketones**

Organism	Ascorbic Acid	Inosose	Dihydroxyacetone	Acetoin	Diacetyl	1,2-Cyclohexanedione	1-Phenyl-1,2-Propanedione
H37Rv strain of *Mycobacterium tuberculosis*	150	250	250	250	3	46	24
BCG strain of *Mycobacterium tuberculosis*	150	200	250	250	3	31	24
Ravenel strain of *Mycobacterium tuberculosis*	375	500	500	500	9	125	62
Mycobacterium phlei	450	625	625	925	15	125	93
Escherichia coli	1,875	2,500	2,500	2,500	31	250	125
Pseudomonas sp	2,500	5,000	5,000	2,500	31	500	250

* Expressed as minimal amount of antibacterial substance in μg/ml necessary for complete inhibition.

and observed 7 and 14 days after inoculation, whereas cultures of *M. phlei*, *E. coli* and *Pseudomonas* sp. were incubated at 37 C and observed after 24 to 48 hours of incubation. The tube containing the least amount of antibacterial substance and showing absence of growth by visual inspection was the end point.

Paper chromatography. Paper chromatography of reductones was carried out and developed according to the procedure of Mapson and Partridge (1949) using butanol-acetic acid-KCN.

Short term growth experiments. Nutrient broth (Difco) was dispensed in 5.5 ml amounts in Coleman cuvettes covered with aluminum foil caps. The antibacterial substances were added and the volume brought to 6.0 ml. The tubes were inoculated with 0.1 ml of a culture of *E. coli* which contained approximately 10^{-2} mg wet weight. Nephelometric readings were made at 30 minute intervals in a Coleman nephelometer model 9.

RESULTS

The possible role of the enediol group was studied by comparing the antibacterial spectra of ascorbic acid and various other compounds known to give rise through autooxidation to enediol containing compounds. Qualitative tests for the presence of the enediol group were performed by placing small spots of solutions on filter paper, allowing them to dry, and spraying them with 0.04 M 2,6-dichlorophenolindophenol. It was found that scyllo-inosose, dihydroxyacetone, acetoin, diacetyl, fructose, and xylose when allowed to stand at pH 7.0 for several days formed reductones[2] demonstrable with this test.

[2] von Euler and Hasselquist (1950) have reviewed much of their work on reductones and have presented data which suggest the pathway by which some of these reductones are formed.

All the reductogenic compounds tested except fructose and xylose had similar antibacterial spectra (table 1). Diacetyl, which according to von Euler and Hasselquist (1950) undergoes a dismutation reaction resulting in one molecule of reductone and two molecules of acetic acid, was considerably more potent than the other reductogenic compounds even though its antibacterial spectrum was essentially identical to the less potent substances. Fructose and xylose apparently formed insufficient "reductone" to inhibit *M. phlei*, *E. coli*, and *Pseudomonas* sp. and are not included in table 1.

The number of reductones formed from the above compounds was determined by chromatographing one per cent solutions which had been adjusted to pH 9, heated under nitrogen for 5 minutes at 90 C, and adjusted to pH 6. Reductones from dihydroxyacetone, inosose, acetoin, and diacetyl were demonstrated and compared with triose reductone,[3] reductic acid,[3] and ascorbic acid. The comparative Rf values are presented in table 2. Several attempts to chromatograph the reductones arising from xylose and fructose were unsuccessful. The results (table 2) make it seem likely that the reductone arising from dihydroxyacetone is triose reductone. The reductones arising from acetoin and diacetyl appear identical as might be expected from the structural relationships.

The possible antibacterial role of the enediol group was next studied employing 5 per cent ascorbic acid solutions in M/30 phosphate buffer at pH 7.0 and allowing them to autooxidize at 37 C for intervals up to 30 days. Aliquots were removed at intervals for assay against the BCG strain and for chromatographic demonstration of

[3] Obtained from Bios Laboratories, Inc., 17 West 60th Street, New York 23, N. Y.

TABLE 2

Comparative Rf values of reductones resulting from the following compounds

	Rf
Fresh ascorbic acid	0.40
Autooxidized ascorbic acid*	0.56
	0.16
Dihydroxyacetone	0.63
Triose reductone	0.63
Acetoin	0.90
Diacetyl	0.90
Reductic acid	0.70
Inosose*	0.37
	0.19

* Formed two reductones.

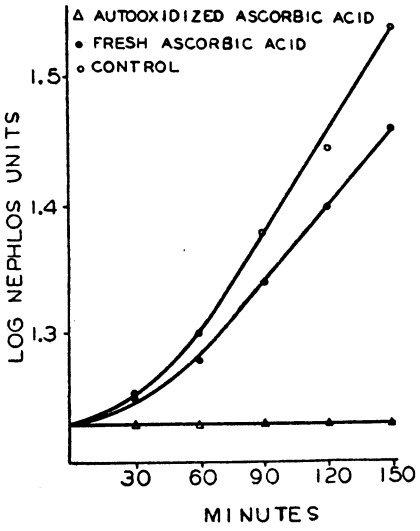

Figure 1. The effect of fresh and autooxidized ascorbic acid on the growth of *Escherichia coli*. The concentration of ascorbic acid was 10 mg/ml in nutrient broth (Difco). Autooxidized ascorbic acid was incubated for 96 hours at 37 C prior to inoculation.

the enediol group. It was noted that the enediol compound corresponding to ascorbic acid disappeared after 2–3 weeks, and two additional unidentified enediol compounds appeared. The last enediol compound to appear persisted long after the 30 days' incubation interval and in one instance was demonstrable for 6 months. The solutions allowed to autooxidize for 30 days were still capable of inhibiting the BCG strain at 100 micrograms per ml in Proskauer and Beck's medium. The persistence of an unidentified enediol derivative from ascorbic acid suggested that the enediol group might be responsible for the antibacterial effects. Since enediol compounds are oxidized by methylene blue which acts as a mediator for atmospheric oxygen, methylene blue was incorporated in the assay medium in concentration of 1 to 1,000,000. Ascorbic acid was added in varying amounts, and the tubes were inoculated with strain BCG. The addition of methylene blue caused no change in the inhibitory concentration of ascorbic acid, namely, 100 μg/ml, even though no reductone could be detected in the tube containing the most ascorbic acid, namely, 1 mg/ml, using the spot test technique. This was taken as evidence that no enediol compounds remained in the zone which inhibited the growth of the BCG strain, which in turn suggested that the enediol group was not responsible for the antibacterial properties. This idea found further support in short term growth experiments that were conducted in the Coleman nephelometer using *E. coli* as the test organism. Freshly prepared ascorbic acid in a concentration of 10 mg per ml in nutrient broth failed to inhibit the growth of *E. coli*. In contrast, when the same concentration of ascorbic acid was incubated at 37 C in nutrient broth for 96 hours before inoculation, *E. coli* was completely inhibited (figure 1). When as little as 200 μg/ml of freshly prepared diacetyl were incorporated into nutrient broth, *E. coli* was immediately inhibited.

The instability of the enediol group and its direct oxidation to a relatively stable alpha diketone group together with the known antibacterial properties (Bloch *et al.*, 1945; Schales, 1951) of alpha diketones prompted a study of the antibacterial spectra of alpha diketones. The antibacterial spectra of 1,2-cyclohexanedione and 1-phenyl-1,2-propanedione were demonstrated to be similar to that of ascorbic acid and diacetyl (table 1). These data indicate that the diketone group possesses antibacterial properties that are very similar to ascorbic acid and diacetyl. Qualitative tests for reductones arising from the diketones 1,2-cyclohexanedione and 1-phenyl-1,2-propanedione were negative. Thus, diketones unable to form enediol derivatives nevertheless possess antibacterial properties that are very similar to autooxidized ascorbic acid.

DISCUSSION

The mechanism and the exact chemical derivative by which ascorbic acid inhibits bacterial

growth remain to be specifically defined. The data presented support the idea that the alpha diketone group (oxidized enediol) is the responsible chemical group. The similarity between the antibacterial spectra of reductogenic compounds, alpha diketones, and ascorbic acid provides support for this proposal. Furthermore, the short term growth experiments with *E. coli* failed to demonstrate any antibacterial properties of the enediol group but did reveal strong immediate antibacterial effects by the diketone diacetyl and by autooxidized ascorbic acid.

One might expect that 2,3-diketogulonic acid would be responsible for the antibacterial effects of ascorbic acid since it is the direct oxidized diketone derivative from ascorbic acid. It has been shown (Penney and Zilva, 1943) that this diketone is very unstable and perhaps could only be inhibitory in a dynamic system. In this regard, Myrvik *et al.* (1954) failed to demonstrate any appreciable tuberculostatic activity with the calcium salt of 2,3-diketogulonic acid. Since one of the reductones persisted for six months in a solution of ascorbic acid, it seems more likely that the diketone derivatives which would arise from the unidentified reductones of ascorbic acid might be mainly responsible for the antibacterial properties of ascorbic acid.

Nickerson *et al.* (1953) have reported that low concentrations of reductone (probably triose reductone) have an inhibitory effect on the growth of *E. coli* but stimulate that of *Micrococcus pyogenes* var. *aureus*. They suggest that reductones are inhibitory by binding cobalt into a cobalt-reductone complex. However, data from this laboratory have demonstrated that fresh ascorbic acid is not inhibitory, thus indicating that it is not the enediol group that is responsible for inhibition, but an oxidized derivative of this group. It is well established (Schales, 1951; Bloch *et al.*, 1945) that alpha diketones possess anti-bacterial properties, including marked tuberculostatic activity. Since enediol groups appear to be universally oxidized to alpha diketones, one might expect that in a dynamic system the concentration of alpha diketone derivatives would be sufficient to explain the antibacterial properties of the reductogenic compounds as well as ascorbic acid undergoing autooxidation.

SUMMARY

The antibacterial spectra of ascorbic acid, inosose, dihydroxyacetone, acetoin, diacetyl, 1,2-cyclohexanedione and 1-phenyl-1,2-propanedione were found to be similar. Qualitative tests for reductones indicated that inosose, dihydroxyacetone, acetoin, and diacetyl were capable of forming derivatives with enediol groups. In addition, it was demonstrated by chromatography that ascorbic acid undergoing autooxidation yielded two reductones which were unidentified.

Short term growth experiments indicated that the enediol group has no antibacterial properties but that the oxidized enediol (diketone) produces immediate bacteriostasis. It is proposed that the diketone groups resulting from the oxidation of enediol groups are responsible for the antibacterial properties of autooxidized ascorbic acid and the reductogenic compounds.

REFERENCES

Bloch, H., Lehr, H., Erlenmeyer, H., and Vogler, K. 1945 Über den Stoffwechsel von Tuberkelbazillen. IV. Der Einfluss von 1,2-Diketonen auf das Wachstum von Tuberkelbazillen. Helv. Chim. Acta, **28**, 1410–1413.

Boissevain, C. H., and Spillane, J. H., Jr. 1937 A note on the effect of synthetic ascorbic acid (vitamin C) on the growth of the tubercle bacillus. Am. Rev. Tuberc., **35**, 661–662.

Ehrismann, O. 1942 Über das Verhalten aerober und anaerober Bakterien gegenuber Askorbinsaure. Z. Hyg. Infektionskrankh., **123**, 16–44.

Grootten, O., and Bezssonoff, N. 1935 La sensibilite du bacille de la coqueluche via-a-vis de la vitamin C et de l'hydroquinol. Compt. rend. soc. biol., **120**, 121–123.

Leitner, St. J. 1937 Der Einfluss von Vitamin C und Vitamin B_1 auf das Wachstum der Tuberkelbacillen. Klin. Wochschr., **16**, 1423–1425.

Lwoff, A., and Morel, M. 1942*a* L'action del vitamine C sur la multiplication de *Proteus vulgaris*. Ann. inst. Pasteur, **68**, 255–258.

Lwoff, A., and Morel, M. 1942*b* Conditions et mechanisme de l'action bactericide de la vitamine C. Role de l'eau oxygenee. Ann. inst. Pasteur, **68**, 323–342.

Mapson, L. W., and Partridge, S. M. 1949 Separation of substances related to ascorbic acid. Nature, **164**, 479–480.

Myrvik, Q. N., Weiser, R. S., Houglum, B., and Berger, L. R. 1954 Studies on the tuberculoinhibitory properties of ascorbic acid derivatives and their possible role in inhibition of tubercle bacilli by urine. Am. Rev. Tuberc., **69**, 406–418.

NICKERSON, W. J., MERKEL, J. R., AND ROLAND, F. 1953 Antibacterial action of an enediol (reductone). J. Infectious Diseases, **93**, 278–281.

PENNEY, J. R., AND ZILVA, S. S. 1943 The chemical behavior of dehydro-l-ascorbic acid *in vitro* and *in vivo*. Biochem. J. (London), **37**, 403–417.

SCHALES, O. 1951 Effect of albumin on the antibacterial activity of diketones. Arch. Biochem. and Biophys., **34**, 56–63.

SIRSI, M. 1952 Antimicrobial action of vitamin C on *M. tuberculosis* and some other pathogenic organisms. Indian J. Med. Sci., **6**, 252–255.

SLADE, H. D., AND KNOX, G. A. 1950 Nutrition and the role of reducing agents in the formation of streptolysin O by a group A hemolytic streptococcus. J. Bacteriol., **60**, 301–310.

VON EULER, H., AND HASSELQUIST, H. 1950 *Redukton. Ihre chemischen Eigenschaften und biochemischen Wirkungen.* Verlag Ferdinand Enke, Stuttgart, 55 pp.

VON GAGYI, J. 1936 Über die bactericide und antitoxische Wirkung des Vitamin C. Klin. Wochschr., **15**, 190–195.

YOUMANS, G. P. 1946 A method for the determination of the culture cycle and the growth rate of virulent human type tubercle bacilli. J. Bacteriol., **51**, 703–710.